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(54) Modified zein.

(57) Modified seed storage proteins of maize (zeins) containing lysine and genetically modified structural genes encoding these proteins. The structural genes encoding the 19kd and 22kd zein proteins have been genetically modified so that the zeins are modified to contain lysine. These modifications are made without affecting the ability of the modified zeins to function correctly as a storage protein in maize endosperm. Various genetic constructs and vectors comprising these modified zein genes are provided.

EP 0 208 418 A2

MODIFIED ZEIN

Seed storage proteins are the major source of protein in a vegetarian diet. Most seed storage proteins are nutritionally incomplete in that they lack one or more of the essential amino acids for proper nutrition of higher animals including man. Reliance on a single source of vegetable protein to meet dietary needs leads to deficiency conditions. For example, the cereal seed storage proteins typically lack the essential amino acid lysine, and overdependence on a cereal, notably corn, for dietary protein is in part responsible for the condition of malnutrition known as kwashiorkor.

When using corn as feed or in the human diet, it is therefore necessary to supplement the diet with another protein source or with lysine itself. Such supplemental feeding is not always convenient or economically feasible. The present invention provides a modified zein which contains lysine. The modified zein serves as a nutritionally balanced source of protein. The modified zein can be provided in any form acceptable to the intended consumer, e.g., as a single cell protein, or as the seed storage protein of a genetically modified plant, which may be maize, soybean, sunflower or other plant species carrying and expressing a gene encoding the modified zein, either in its seeds or other edible tissues.

Maize Zein Proteins

The storage proteins of maize seed consist of a group of prolamine proteins called zeins. (For review, see Larkins, B., Genetic Engineering of Plants (T. Kosuge, C. P. Meredith and A. Hollander eds.) Plenum, New York 1983, pp. 93-118.) The zeins are water-insoluble, but soluble in alcohol. Zein proteins are synthesized by membrane-bound polyribosomes in the developing endosperm and are deposited as aggregates called protein bodies within the rough endoplasmic reticulum (RER). Because of this, one observes RER membranes surrounding protein bodies in electron micrographs of maize

endosperm. It is often difficult to observe continuity between membranes surrounding the protein bodies and the RER, but previous studies have shown the existence of similar populations of polyribosomes on the surface of both membranes (Larkins, B. A. and W. J. Hurkman (1978) *Plant Physiol.* 62:256-263). Perhaps the most convincing evidence that zein protein bodies form simply by protein aggregation within the RER is the observation that structures with the same physical characteristics as protein bodies can be isolated from Xenopus laevis oocytes previously injected with zein mRNAs (Hurkman et al. (1981) *J. Cell. Biol.* 89:292-299).

A 2-dimensional gel analysis of the alcohol-soluble proteins contained within the protein bodies reveals a mixture of polypeptides. The most abundant of these have apparent molecular weights of 22,000 and 19,000, but there are also some smaller polypeptides of 15,000 and 10,000 daltons. There is significantly more charge heterogeneity among the Mr 22,000 and Mr 19,000 zeins than the small molecular weight zein proteins.

Marks and Larkins (1982) *J. Biol. Chem.* 257:9976-9983; and Pederson, et al. (1982) *Cell* 29:1015-1026 have constructed cDNA clones of zein mRNAs and determined the DNA sequence for representative Mr 22,000, Mr 19,000, and Mr 15,000 zein proteins. The sequence for an Mr 19,000 zein has also been reported by Geraghty et al. (1981) *Nucleic Acids Res.* 9:5163-5174. From knowledge of the DNA sequence it has been possible to determine the complete primary amino acid sequence of the polypeptides and compare them for structural similarities.

This analysis revealed that the zeins were significantly larger than expected based on their mobility on SDS polyacrylamide gels. Zein proteins that had been estimated to have molecular weights of 22,000 and 19,000 were found to be closer to 27,000 and 23,000, respectively (Table I). This analysis also confirmed the presence of signal peptides on the zein proteins (Fig. 1). These signal sequences were previously demonstrated to be removed when the protein is transported into the lumen of the RER (Larkins et al. (1979) *Proc. Natl. Acad. Sci. USA* 76:6448-6452).

Amino Acid Composition of Maize Zein Proteins

Apparent Molecular Weight	^a Zein α Mr 22,000	^b Zein β Mr 19,000	Zein Mr 15,000
<hr/>			
Amino Acid			
Leu	42	44	15
Gln	41	39	28
Ala	34	31	18
Pro	22	21	13
Ser	18	15	11
Phe	8	13	0
Asn	13	9	2
Ile	11	9	1
Tyr	6	8	16
Val	17	6	4
Gly	2	4	12
Thr	7	4	5
Arg	4	3	7
His	3	3	4
Cys	1	2	6
Glu	1	1	5
Met	5	1	11
Asp	0	1	4
Lys	0	0	0
Trp	0	0	1
<hr/>			
TOTAL	225	214	163

^a Marks and Larkins (1982) J. Biol. Chem. 257:9976-9983

^b Pedersen et al. (1982) Cell 29:1015-1026

The amino acid composition predicted from the polypeptide sequence is similar to that previously found for mixtures of zein proteins (Lee et al. (1976) Biochem. Genet. 14:641-650). Glutamine, leucine, proline, alanine, and glycine account for the majority of the amino acids, and lysine and tryptophan absent from all of them (Table I). It is interesting to note that methionine, which is deficient in most legume storage proteins, accounts for a significant percentage of the Mr 15,000 zein. In fact, cysteine and methionine account for 11% of the total amino acids in this polypeptide.

A particularly interesting feature of the protein sequence is the occurrence of a conserved, tandemly repeated peptide in both of the Mr 22,000 and Mr 19,000 zeins (Fig. 1). The first of these repeat sequences begins 35-36 amino acids after the NH₂-terminus, and is repeated nine times in each polypeptide. The COOH-terminal sequence following the repeats is slightly longer in the Mr 22,000 zeins; this accounts for the size difference between the two polypeptides. Most of the amino acids in these repeats are nonpolar, while the repeated peptide is sequentially polar, nonpolar, polar, nonpolar, polar.

Circular dichroism measurements of mixtures of zein proteins indicate from 45-55% α -helical structure (Argos et al. (1982) J. Biol. Chem. 257:9984-9990) and this percentage correlates well with the proportion of amino acids in these repeated peptides. To determine if the repeats have the potential to form α -helices we compared their amino acid sequences with those found in proteins having α -helical structure (Argos et al., supra) Although a comparison to soluble proteins shown little propensity for these repetitive sequences to be α -helical, they do have α -helical properties when compared with sequences found in some hydrophobic proteins. In view of the hydrophobic nature of zein proteins, it seems reasonable to predict an α -helical structure for them.

Assuming that these are α -helices, a model showing how the nine repeats could be organized into a 3-dimensional structure has been published. The model predicts that when the consensus repeat is placed in an α -helical wheel the polar amino acids are distributed at three symmetrical sites. Considering that the repeats are tandem, if they fold back upon one another in an antiparallel arrangement, two polar groups in each repeat can hydrogen bond

with each of two adjacent repeats. The nine helices would then interact to form a roughly cylindrical, rod shaped molecule. The cylinder would collapse in the center to accommodate the non-polar tails of the amino acids. As these protein molecules associate within the endoplasmic reticulum, the third polar group, which is on the surface of the helix, would hydrogen bond to a different zein molecule. This arrangement also allows the glutamine residues, which lie at the ends of the helices, to hydrogen bond with neighboring protein molecules in the protein body. Fig. 7 provides a two-dimensional representation of the proposed model.

The published model explains many of the physical properties of the proteins (Argos *et al.*, supra), although it has not been confirmed by x-ray diffraction patterns of protein crystals. If the interaction of these α -helices is important in structuring the polypeptide and aggregating it into a protein body, it would appear that altering the amino acid sequence of these repeated regions could deleteriously affect the protein's structure. Larkins (1982) supra, stated that it would seem more advantageous to change the NH_2^- terminal or COOH -terminal turn sequences which lie outside the repeat structures.

SUMMARY OF THE INVENTION

The invention includes modified 19 kd and 22 kd zeins containing lysine, modified structural genes encoding protein precursors to said modified zeins and various genetic constructs and vectors comprising these modified genes. The modified zeins resemble normal unmodified zeins in their molecular weights, ability to form protein bodies within the rough endoplasmic reticulum of a host cell, and solubility in alcohol.

DETAILED DESCRIPTION OF THE INVENTION

Conventional nomenclature for the zeins refers to their nominal molecular weights based on mobility on SDS polyacrylamide gels. Although the actual molecular weights, deduced from the nucleotide sequences of the genes encoding these proteins is somewhat larger, as stated supra, the proteins will be referred to in terms of the conventional nomenclature. Zeins of molecular weight of approximately 15 kd, 19 kd and 22 kd have been reported. The

present invention relates to the 19 kd and 22 kd zeins. The secondary and tertiary structures of the 19 kd and 22 kd zeins is very similar, the primary difference being that the larger zeins have an extended COOH-terminal sequence. The principles of amino acid substitution are exemplified herein in connection with a 19 kd zein. Similar modifications can be made in the 22 kd zeins without substantially effecting their zein-like properties.

An important operating principle maintained throughout is that a modification must not interfere with the ability of the modified protein to function as a storage protein in maize endosperm. The unusual solubility properties of zein, the ability to be translocated within the cell and the ability to form characteristic protein bodies are considered important criteria for correct functioning of a modified zein protein. Hurkman *et al.* (1981) have demonstrated that the foregoing criteria can be measured in the frog oocyte expression system. Thus, authentic pre-zein mRNA can be injected into frog (*Xenopus laevis*) oocytes wherein translation yields a protein which accumulates in rough endoplasmic reticulum in protein bodies resembling those found in maize endosperm tissue. The term "modified zein" is therefore reserved for zein proteins having an amino acid sequence which is not naturally occurring, which behaves similarly to authentic zein in a frog oocyte expression system and which is soluble in alcohol. The term "pre-zein" denotes the primary translation product of mRNA comprising a zein coding sequence, said primary translation product comprising an additional NH₂-terminal sequence not found in mature zein isolated from protein bodies. The additional NH₂-terminal sequence is believed to function as a signal peptide which aids in intracellular compartmentalization of the translation product. The terms "modified pre-zein" and "modified pre-zein gene" as used herein have the same meanings with respect to the modified sequences as those used in connection with the unmodified sequences, the only difference being related to changes in the primary amino acid sequences described herein.

An important feature of the present invention is based upon the unexpected finding that the substitution of a lysine within one of the repeated segments of the molecule yields a modified, lysine-containing zein product that meets the functional and physical criteria described, *supra*. Also contrary to expectation, the substitution of a lysine for an amino acid within the unrepeated NH₂-terminal portion of the molecule yields a product

which does not meet the above-described criteria. The invention has been exemplified by the modification of a DNA encoding a 19 kd pre-zein gene such that the coding sequence encodes a lysine in the place of an amino acid of the naturally-occurring sequence.

In the examples, the conversion of an asparagine residue to lysine at a locus within the repeated portion of the sequence yielded a lysine containing modified zein which behaved normally when translated in frog oocytes. On the other hand, conversion of an isoleucine residue to lysine in the NH₂-terminal turn of the mature polypeptide did not yield a detectable protein having the properties of a zein as judged by the before-mentioned criteria. The results demonstrate that, contrary to expectation, substitution of a lysine for an amino acid in the repeated portion of the molecule is tolerated without detrimental effect to zein function and properties whereas, again contrary to expectation, a substitution outside the repeated region, at the NH₂-terminal end of the molecule, results in a product that apparently lacks zein-like properties. Although the results speak for themselves and the invention does not depend on any hypothesis or theory of operation, the results are consistent with observations in other systems where complete translation had been shown to depend upon coupled, translocation to endoplasmic reticulum, which in turn depends on the integrity of the NH₂-terminal sequences of pre-protein and of mature protein. It will be understood by those of ordinary skill in the art, following the teachings of the present invention, that modifications other than those exemplified can be used to introduce a desired modification in amino acid composition while retaining the functional and physical properties of zein. Amino acid substitutions other than lysine for asparagine, especially those which do not substantially modify the hydrophobicity of the repeated regions may be introduced. An important operating principle is to preserve the overall hydrophobic character of the internal repeats so as to preserve their character as domains responsible for establishing the tightly folded configuration of the zein molecule. A second principle is to retain the conformation and hydrophobicity of the NH₂-terminal sequences of modified pre-zein and zein, in order to retain the function of correct translocation in the host cell. Since lysine is protonated at intracellular pH levels, lysine replacement of a highly non-polar amino acid is likely to be more disruptive than replacement of a polar, or moderately

non-polar amino acid. In addition, amino acid residues that are attractive candidates for replacement will be located in less conserved regions of the repeated sequences. Such less conserved (divergent) regions can be identified by comparisons of several 19 kd or 22 kd zein sequences. Besides amino acid substitution within the repeated sections, single amino acid insertions can be employed, as well as substitutions in the segments connecting the domains of repeated sequences. Also, insertions and substitutions can be made in the COOH-end of the zein molecule. In particular, the substitution of a lysine for a glutamine within the segment postulated to connect two repeated segments yields a structure having the required properties. An upper limit to the number of lysines which may be introduced into a modified 19 kd or 22 kd zein has not been determined. A determination of an upper limit is not critical, however, because as little as one lysine residue per modified zein molecule is sufficient to confer a valuable nutritional benefit. Regulation of the amount of lysine produced by cells or tissues expressing a modified zein coding sequence can be achieved in one of two ways: by increasing the number of lysine residues per molecule or by increasing the number of molecules synthesized per cell. The latter can be achieved according to well known principles in the art, using an appropriate promoter whose activity is sufficient to insure the desired level of gene expression in the desired host cell.

Means for modifying a 19 kd or 22 kd zein structural gene are well known in the art and may be employed according to well known and understood principles in the art to achieve the desired modification. The method of site-specific mutagenesis, using M13-based single stranded DNA vectors and synthetic oligonucleotide primers is exemplified herein, but other means for effecting the desired coding sequence changes may be employed. The technique of site-specific mutagenesis employs the chemically synthesized oligonucleotide corresponding to the coding segment whose modification is desired, with a base substitution at the exact site where a change in the coding sequence is desired. The synthetic oligonucleotide is allowed to hybridize with single stranded DNA comprising its complement, then used to prime DNA synthesis of a new coding strand incorporating the desired base change. When the technique is being used to alter a sequence lying within a region known to contain repeats, care must be taken to insure that the

oligonucleotide binds to the template at the desired site. The oligonucleotide must be either sufficiently unique or sufficiently long to prevent mispairing.

The DNA encoding native, or unmodified, zein may be cloned as cDNA or genomic DNA. Although maize contains a number of genes encoding both 19 kd and 22 kd zeins, none has been found to date which contain introns.

Consequently, the modification of cDNA is an entirely feasible strategy even if the end result desired is to reincorporate the modified coding sequence into the maize genome to produce genetically modified maize containing nutritionally balanced zein.

A variety of systems for expressing modified zein can be used. A structural gene encoding modified pre-zein may be combined with a promoter known to provide adequate levels of expression in the chosen host cell. The host cells may be any host cell in which expression of modified zein is or can be made compatible, including bacteria, fungi, yeasts, plant cells and animal cells. For example, a modified pre-zein structural gene as exemplified herein can be combined with the phaseolin promoter, which is known to provide tissue-specific expression in plants (Sengupta-Gopalan *et al.* Proc. Natl. Acad. Sci. USA, in press). The composite gene thus constructed can be introduced to plant cells of a desired species using any of a variety of vectors comprising the T-DNA of Agrobacterium tumefaciens Ti plasmid (See Caplan, A. *et al.* (1983) Science 222:815; Barton, K. A. and Chilton, M.-D. (1983) Meth. Enzymol. 101:527; and Leemans, J. *et al.* (1982) in Molecular Biology of Plant Tumors (G. Kahl and J. Schell, eds.) Academic Press, NY, p 537). Depending upon the means used for introducing the composite gene into plant cells, and the host plant cell species chosen, the cells receiving the composite gene can be regenerated to form fertile adult plants according to known techniques. Seeds of the regenerated plant will express the modified zein thereby providing a nutritionally balanced diet with respect to amino acid composition for humans or animals using the seeds of the plant as food. Current techniques for gene transfer and regeneration are available for such agronomically significant crops as tomato, soybean, sunflower and a number of vegetable crops. Furthermore, the number of crops in which gene transfer and regeneration techniques are being developed is continuously increasing. Expression of a modified zein need not be confined to seed or endosperm tissue. In some instances it will be desirable to enhance the nutritional value of leaves,

stems and other edible vegetative tissues of a plant, to enhance the nutritional value of the plant.

In addition to plants, other organisms, especially single celled organisms, may be genetically altered to produce a modified zein, for example as a convenient form of single cell protein. The properties of zein which make it useful for storage of protein in a highly concentrated and stable form in the maize endosperm are expected to be of value for the production of highly nutritious single cell protein.

Modified pre-zein genes may also be expressed in animal cells. In fact, an animal cell system, frog oocytes, was employed as detailed in the examples, for the purpose of rapidly determining whether a given genetic modification yields a modified zein having the appropriate functional and physical characteristics. In the examples, a modified pre-zein coding segment was cloned into the E. coli vector SP6. Recombinant SP6, carrying the inserted pre-zein coding segment, was used as template in an in vitro RNA polymerase-catalyzed reaction to produce messenger RNA encoding modified pre-zein. The RNA preparation was then injected into frog oocytes which, when incubated in an appropriate medium under known techniques and conditions, are capable of translating the injected mRNA. The synthesis of zein or modified zein, as the case may be, was measured by the appearance of a protein band on an electrophoresis gel, after extracting alcohol soluble, aqueous insoluble protein from the oocytes. The appearance of a protein band at a position corresponding to 19 kd or 22 kd, as the case may be, indicated the synthesis of zein, or modified zein by the oocytes. In a second type of assay, the microscopic appearance of protein bodies in the rough endoplasmic reticulum of oocytes was observed in oocytes expressing native zein or zein modified according to the invention.

In the course of the experiments described in the examples, it was observed that cDNA which had originally been cloned using the technique of homopolymer tailing resulted, after transcription using the SP6 system, in messenger RNA which was poorly translated. Translation was substantially enhanced by pretreating the cDNA with limited exonuclease digestion to remove the homopolymer tails, prior to cloning into SP6.

II

The following examples further illustrate the invention. Except as noted hereafter, standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art (See, for example, R. Wu, ed. (1979) Meth. Enzymol. 68; R. Wu et al., eds. (1983) Meth. Enzymol. 100, 101; L. Grossman and K. Moldave, eds. (1980) Meth. Enzymol. 65; J. H. Miller (1972) Experiments in Molecular Genetics; R. Davis et al. (1980) Advanced Bacterial Genetics; R. F. Schleif and P. C. Wensink (1982) Practical Methods in Molecular Biology; and T. Maniatis et al. (1982) Molecular Cloning). Abbreviations, where employed, are those deemed standard in the field and commonly used in professional journals of wide circulation such as those cited herein.

Textual use of the name of a restriction endonuclease in isolation, e.g., "BclI" refers to use of that enzyme in an enzymatic digestion, except in a diagram where it can refer to the site of a sequence susceptible to action of that enzyme, e.g., a restriction site. In the text, restriction sites are indicated by the additional use of the word "site", e.g., "BclI site". The additional use of the work "fragment", e.g., "BclI fragment", indicates a linear double-stranded DNA molecule having ends generated by action of the named enzyme (e.g., a restriction fragment). A phrase such as BclI/SmaI fragment" indicates that the restriction fragment was generated by the action of two different enzymes. Note that the ends will have the characteristics of being either sticky (i.e., having a single strand of protrusion capable of base-pairing with a complementary single-stranded oligonucleotide) or blunt (i.e., having no single-stranded protrusion) and that the specificity of a sticky end will be determined by the sequence of nucleotides comprising the single-stranded protrusion which in turn is determined by the specificity of the enzyme which produces it.

Example 1: Cloning of double-stranded cDNAs

Zein mRNAs were isolated from membrane-bound polyribosomes of the maize inbred W64A as described by Larkins and Hurkman (1978) and used as templates for double-stranded (ds) cDNA synthesis as described by Buell *et al.* (1978). S1 nuclease-treated ds-cDNAs were fractionated on a 5% polyacrylamide slab gel. Gel slices that contained cDNAs of 700 or more bases were placed in dialysis bags filled with 1 ml of 0.5X TBE (TBE = 0.01 M Tris, pH 8.3, 0.01 M boric acid, 0.01 mM EDTA) and were subjected to an electric field of 50 volts for 2 hours. The ds-cDNA was recovered by ethanol precipitation in the presence of 0.3 M Na acetate. Homopolymer tails of oligo (dC) were added to the ds cDNA using terminal transferase as described by Roychoudhury *et al.* (1976) and the ds cDNA was annealed to the plasmid pUC8 that had been homopolymer tailed with oligo (dG). The recombinant plasmids were used to transform the bacterial strain JM83 that was made competent by the method of Morrison (1979). Bacteria containing recombinant plasmids were identified by the filter hybridization procedure of Grunstein and Hogness (1975) using ³²P-labeled cDNA as a probe. By using then pUC 8-JM83 transformation system (Messing and Vierira, 1982), a 10-fold increase was obtained in cloning efficiency over the pBR 322-HB101 system that was previously used.

The sequences of several 19 kd and 22 kd cDNA clones are shown in Fig. 2 and Fig. 3, respectively. The corresponding amino acid sequences are shown in Figs. 4 and 5.

Figure 2 shows the nucleotide sequence of clones for the 19 kd zeins. The nucleotide sequence of gZ19AB1 (previously named ZG99) was previously reported (Pederson *et al.*, 1982). The complete sequences of gZ19AB1, cZ19D1, and cZ19C2 are given. The sequences of the cZ19A2 and cZ19B1 follow the colons and only nucleotides in the sequences that vary from gZ19AB1 are shown. Likewise, the cZ19C1 sequence begins after the colon and only nucleotides in that sequence which vary from cZ19C2 are shown. "An" indicates a sequence terminating in a poly(A) tail. Asterisks indicate positions where gaps were introduced in the sequences to maximize homology. The putative polyadenylation signals are underlined. Positions corresponding to the initiating methionine (Met) and the NH₂-terminus and -COOH terminus of the encoded polypeptide are indicated.

Figure 3 shows the nucleotide sequence of clones for the 22 kd zeins. Nucleotides are numbered starting with the first base of the initiator codon and are indicated on the right-hand margin. The sequences of cZ22A1 and cZ22B1 (previously named pZ22.1 and pZ22.3, respectively) were previously reported (Marks and Larkins, 1982). The complete nucleotide sequence of cZ22A1 is given, but only variable nucleotides at corresponding positions are listed for the other clones. Asterisks indicate positions where gaps were introduced in the sequence to maximize homology. The positions of the first nucleotides for cZ22B-1 and cZ22C-2 follow the colons. "An" indicates the position at which a sequence terminates in a poly(A) tail. Positions corresponding to the initiating methionine (Met) and the NH₂-terminus and -COOH terminus of the encoded polypeptide are indicated.

Figure 4 shows amino acid sequences deduced from the DNA sequence of clones for the Mr 19,000 zeins (Fig. 2). The complete amino acid sequences deduced from gZ19AB1, cZ19D1, and cZ19C1 are given. Only amino acid residues for cZ19A2 and cZ19B1 that differ from gZ19AB1 are shown as are only amino acid residues for cZ19C2 that differ from the sequence of cZ19C1. Asterisks indicate gaps introduced in the amino acid sequence to demonstrate homologies. The positions of the NH₂-terminus and -COOH terminus of the mature proteins are indicated.

Figure 5 shows amino acid sequences deduced from DNA sequences of clones for the Mr 22,000 zeins (Fig. 3). The complete sequence of cZ22A1 is given in the standard single letter amino acid code, but only variable amino acid residues at corresponding positions are listed for the other sequences. The position corresponding to the NH₂-terminus of the mature protein is indicated.

Example 2: Removal of homopolymer tails and cloning in M13

In order to generate efficiently translatable mRNA transcripts of a pre-zein gene or modified pre-zein gene it was discovered that the cloned sequences must be further tailored by removing the homopolymer tails generated during the cDNA cloning process. It was found to be sufficient to remove the homopolymer tail at the 5' end of the pre-zein coding sequence.

For this purpose the pUC8 vector containing the 19 pre-zein coding sequence was cut with EcoRI endonuclease. The EcoRI site of pUC8 lies within the same polylinker as the PstI site into which the pre-zein cDNA was originally cloned. Cleavage with EcoRI cuts the vector at the 5' end of the pre-zein gene. The EcoRI linearized plasmid was incubated with Bal31 exonuclease according to standard techniques for varying degrees of limited digestion. The digested linear plasmid was reisolated and cut with HindIII endonuclease. The HindIII site of the pUC8 polylinker lies on the 3' side of the pre-zein gene. The resulting HindIII cleavage releases the pre-zein DNA coding insert in the form of a population of molecules having varying numbers of nucleotides removed from the 5' end.

The resulting population of 5'-deleted inserts was then cloned into M13mpl1, previously cut with SmaI and HindIII to generate a unidirectional cloning. Upon ligation of the inserts into SmaI/HindIII cut M13mpl1, the sticky ends generated by HindIII cutting at the 3' end of the insert anneal with the HindIII cut end of the M13 vector while the blunt ends of the insert generated by Bal31 digestion can ligate by blunt end ligation to the SmaI cut end of the M13 vector.

Clones of individual inserts in M13mpl1 were then sequenced at the 5' end of the insert to determine the amount of homopolymer tail removed by the Bal31 digestion. Three clones, representing three different degrees of digestion, were analyzed for ability to provide efficient translation, using the SP6 transcription system and frog oocyte translation system described infra in Example 4. The highest yield of zein was produced from transcripts derived from cDNA in which the entire 5' homopolymer tail had been removed as well as an additional two nucleotides of the 5' untranslated region of the pre-zein cDNA. Intermediate yields of zein were obtained using clones in which the homopolymer tails were partially removed. Therefore, removal of the homopolymer tail at the 5' end is of substantial importance for efficient translation.

Example 3: Modification of pre-zein coding region by site-specific mutagenesis

M13 bacteriophage and reagents are available as a kit from Amersham Corp., Arlington Heights, Illinois, together with a handbook of instructions for carrying out various manipulations and isolations of single stranded and RF (double stranded) forms of M13 DNA. The handbook of instructions is hereinafter referred to as the "Amersham handbook". E. coli GM119 (dcm-6, dam-3, metB-1 thiI, lac Y1 or lac 4Z, gal K2, gal T22, mtl-2, ton A2 or ton A31, tsx-1 or tsx-78, supE44, malR/F1KM) is a widely available E. coli strain used herein for the purpose of preparing non-methylated M13 DNA. The use of non-methylated DNA favors retention of the mutagenized strand during replication primed by a synthetic oligonucleotide as described, infra.

A culture of E. coli GM119 was grown from a single colony to an O.D. of 0.6 at 32°C in LB medium. Fifty μ l of the O.D. 0.6 culture was added to 2 ml prewarmed LB medium and 5 μ l of M13 phage suspension was added. The culture was grown overnight or for approximately 16 hours at 32°C. After the incubation, intact cells were removed by centrifugation. The supernatant contained phage from which single stranded M13 template DNA was prepared as described in the Amersham handbook.

Gapped heteroduplex DNA comprising unmethylated (+) (viral) strand and a methylated (-) strand was prepared by an annealing procedure. Minus (-) strand DNA was prepared from M13 RF DNA digested with HindIII and EcoRI endonucleases. The reaction mixture was extracted once with a Tris-EDTA buffer saturated with a chloroform/phenol mixture (equal parts by volume). The aqueous phase was then extracted twice with a 5-fold volume of diethyl ether, 0.2 volumes of 5 mM ammonium acetate and 2.5 volumes of ethanol were added to precipitate DNA during a 10-15 minute incubation in a dry ice-ethanol bath. The precipitate was collected by centrifugation, the supernatant was decanted and the tube containing the precipitate was inverted and allowed to air dry. The dried pellet was redissolved in 20 μ l of Tris-EDTA buffer. The annealing reaction was initiated by combining 0.5 μ g of (+) strand viral DNA (unmethylated) and 0.2 μ g linearized RF DNA in 20 μ l of 1x SSC. The mixture was overlayed with 50 μ l of mineral oil and placed in a boiling water bath for 2 minutes. Immediately thereafter, the tube was transferred to a 65° bath and

incubated for 4 hours or more to allow annealing to occur between the (+) and (-) DNA strands. Following annealing, the aqueous layer was transferred to a new tube and traces of mineral oil removed by two successive extractions with 5 volumes of diethyl ether. Residual ether was removed by applying mild vacuum conditions for 2 minutes. The DNA was precipitated by adding 50 μ l cold ethanol to the reaction mixture and incubating the mixture for 20 minutes in dry ice-ethanol, collecting the precipitated DNA by centrifugation and air drying as before. The above procedure yields from 0.1 μ g to 0.3 μ g gapped heteroduplex DNA.

The mutagenic oligonucleotides were prepared according to standard techniques of DNA synthesis. Two mutagenic oligonucleotides were prepared, the first having the sequence GGTAGTTACTTCGAACT intended to provide an isoleucine to lysine change at amino acid position 44 of cZ19C1, and the second having the sequence GTAAATTTGTTAACCG, intended to encode the substitution of lysine for asparagine at amino acid position 167 in cZ19C1. The air dried heteroduplex mixture was dissolved in 1.5 μ l of solution "A" [described by Zoller and Smith, Methods in Enzymology Vol. 100, p. 46B (1983)] and 2 μ l containing approximately 15 pMole of mutagenic oligonucleotide was added, followed by 7.5 μ l of DEPC treated water. (DEPC (Diethylpyrocarbonate) added at about 0.01% (v/v) inactivates RNase and hydrolyzes to yield traces of ethanol and CO₂.) The reaction was heated to 65°C and incubated at that temperature for 15 minutes. The mixture was then annealed by transfer to room temperature for 20 minutes. To 10 μ l of the mixture treated as described, the following were added: 1 μ l each of 20 mM solutions of dATP, dCTP, dGTP and dTTP, 1.2 μ l of 10 mM ATP, 2.5 μ l of 200 mM dTT (dithiothreitol), 1 μ l DNA polymerase Klenow fragment and 1.2 μ l of DEPC treated water. The mixture was incubated for 30 minutes at 22°C (room temperature). Following incubation 50 units of T4 DNA ligase was added and the mixture again incubated at 22°C (room temperature) for a period of 4 hours. The foregoing procedure provided closed circular DNA comprising (+) (viral) strands incorporating the mutated sequence derived from the mutagenic oligonucleotides.

The ligated heteroduplex mixture was used to transform E. coli JM103 cells, rendered competent for transformation according to the procedure described in the Amersham handbook. E. coli JM103 is widely available from a variety of sources. Two hundred μ l of competent cells were mixed with 1 μ l, 2

μ l, 5 μ l or 10 μ l of heteroduplex mixture, incubated on ice for 40 minutes, then heat shocked by transfer to 42°C for 2 minutes, then returned to the ice bath. To each tube was then added 10 μ l 100 mM IPTG and 50 mM 2% (w/v) X-gal, 100 μ l of JM103 cells to provide a bacterial lawn, and 3 ml of 0.7% (w/v) fresh top agar, the latter kept melted at 50°C. The components of each tube were then quickly mixed and poured onto fresh petri plates containing M9 agar. After overnight incubation plaques were formed which appeared white, or colorless, for those phage containing an inserted zein gene.

The efficiency of the procedure yields from 4% to 10% of mutagenized sequences. Clones in which the mutated sequence have been incorporated were identified by the introduction of a new HindIII site in one case, or the loss of a pre-existing HindIII site in the other case, such that a rapid screening could be undertaken without the need for sequence determination of each clone. The restriction pattern of fragments produced by HindIII cleavage was compared after electrophoretic separation with the pattern of fragments produced by unmodified DNA. The resulting modified pre-zein coding sequences were removed from the M13 vector by an EcoRI-HindIII cut and cloned into the transcription vector SP6 (commercially available, e.g., from ProMega Biotech, Madison, Wisconsin) previously digested with EcoRI and HindIII endonucleases.

Example 4: Transcription and translation of modified pre-zein coding segment

The modified pre-zein coding segment, described in Example 3, was released from the M13 vector by digestion with EcoRI and HindIII endonucleases, and cloned into plasmid SP6, cut with EcoRI and HindIII. The transcription plasmid SP6 is commercially available from ProMega Biotec, Madison, Wisconsin. Other reagents and enzymes used for SP6 transcription are also available from ProMega Biotec. The modified pre-zein gene was transcribed from the SP6 promoter in a reaction containing the following: 40 μ l Tris-HCl pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 1 unit/ml RNAsin (trademark ProMega Biotec, Madison, Wisconsin) 100 μ g/ml bovine serum albumin, 0.5 mM ATP, 0.5 mM TTP, 0.5 mM CTP, 0.1 mM GTP, 0.5 mM 7mGpppG and 15 units SP6 RNA polymerase. After 30 minutes incubation at 40°C, 5 additional units of SP6 polymerase and GTP to form a concentration of 0.5 mM were added. The reaction was continued for 1 hour at 40°C. The DNA template was removed by first adding RNAsin to the final concentration of 1 μ g/ μ l and

then adding RNase I to 20 µg/ml final concentration and incubating at 37° for 15 minutes. Under these conditions, approximately 0.2 µg of mRNA was transcribed per µg of plasmid DNA.

Translation of modified pre-zein mRNA was carried out in frog oocytes microinjected with mRNA as described by Hurkman *et al.* (1981). Translation was monitored by incorporation of tritiated leucine into protein after microinjection of mRNA. Membrane fractions were isolated from the oocytes and ethanol soluble proteins extracted and analyzed by electrophoresis on SDS acrylamide gels. The results are shown in Figure 6. Lane A shows that unmodified 19 kd zein mRNA was found as a single band corresponding to about 19 kd as judged by the migration of a series of markers of known molecular weight, shown in lane C. The result shows that unmodified zein was located within the membrane fraction of the oocytes and behaved as an ethanol soluble protein. Lane B showed the results with a modified pre-zein, having an isoleucine to lysine substitution at amino acid No. 44. No protein was found, indicating that the protein, if produced, did not have a structure allowing it to be translocated to endoplasmic reticulum. A control experiment in which the modified pre-zein mRNA encoding the same amino acid substitution was used in an *in vitro* translation system revealed that a protein of the proper length was obtained. Therefore, failure to detect product in the oocyte system was not due to a defect in the modified zein mRNA, since the latter was translated normally in an *in vitro* system. In lane D, oocytes were injected with a modified pre-zein mRNA in which an asparagine residue was replaced by a lysine at amino acid position 167. The result indicates normal translation, translocation and physical properties similar to unmodified zein by all the criteria measured by the experiment.

The sequence and postulated folded configuration of modified and unmodified Z19C1 zein are shown in Fig. 7. The amino acid abbreviations are shown in Table 2. Cross-bars show hydrogen bonds between residues on adjacent domains of α -helix, postulated to stabilize the highly compact central position having repeated segments of amino acid sequence. Asterisks show the positions where lysine was substituted, as described in the foregoing examples. The compactness of the tertiary structure can be more fully appreciated by rolling the figure into a cylinder around the long axis of the page.

While the invention has been exemplified by a modified 19 kd zein containing lysine, other zeins within the group of 19 kd and 22 kd zeins may be modified, lysine may be introduced by other means, at other locations, by additions and insertions as well as by substitutions within the amino acid sequence, all according to the teachings herein and expedients known to those of ordinary skill in the art mutatis mutandis. Such modifications and variations leading to a modified 19 kd or 22 kd zein comprising a lysine residue are deemed to fall within the scope of the invention, as set forth in the claims.

TABLE 2

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AMINO ACID ABBREVIATIONS

A = Ala = Alanine	M = Met = Methionine
C = Cys = Cysteine	N = Asn = Asparagine
D = Asp = Aspartic Acid	P = Pro = Proline
E = Glu = Glutamic Acid	Q = Gln = Glutamine
F = Phe = Phenylalanine	R = Arg = Arginine
G = Gly = Glycine	S = Ser = Serine
H = His = Histidine	T = Thr = Threonine
I = Ile = Isoleucine	V = Val = Valine
K = Lys = Lysine	W = Try = Tryptophan
L = Leu = Leucine	Y = Tyr = Tyrosine

CLAIMS

1. A modified 19 kd or 22 kd zein, the amino acid composition of which comprises lysine.
2. Modified zein according to claim 1 wherein that 5 lysine is located within an internal repeated region of the zein or in a COOH-terminal non-repeated region of the zein.
3. Modified zein according to claim 1 or claim 2 having a structure permitting the modified zein to be 10 translocated to endoplasmic reticulum in a host cell.
4. Modified zein according to any preceding claim in a protein body in the endoplasmic reticulum of a cell or tissue.
5. Modified zein according to claim 1 wherein a lysine 15 is substituted for asparagine at amino acid position 167 in Z19C1.
6. A DNA segment encoding a modified 19 kd or 22 kd pre-zein, the coding sequence of the DNA comprising a codon for lysine.
- 20 7. A DNA sequence according to claim 6, the transcription and translation of which yields a zein located within protein bodies of the endoplasmic reticulum of said host cell.
8. A DNA segment according to claim 6 encoding Z19C1 25 having a lysine substituted for asparagine at amino acid position 167 in Z19C1.
9. A plant, the DNA of which includes a DNA segment according to any of claims 6 to 8.
10. A seed of a plant according to claim 9.
- 30 11. A method of producing a modified zein comprising rearing a plant according to claim 9 and harvesting the seeds thereof.
12. A microorganism or an animal cell, the DNA of which includes a DNA segment according to any of claims 6 to 8.

13. A method of producing a modified zein comprising culturing a microorganism or an animal cell according to claim 12.

19

0208418

Figure 1

Consensus Amino Acid Sequence of Zein Proteins

Zein protein	Signal Sequence	Sequence positions
Z22	MATKILSLLALLALFASATNA	1-21
Z19	MAAKIFCLIMLLGLSASAATA	1-21

N-Terminal Turn

Z22 S I I P Q C S L A P . S S I I P Q F L P P V T S M A F E H P A V Q A Y R 22-55
719 S I F P O C S Q A P I A S L L P P Y . S P A M S S V C E N P I L L P Y R 22-57

Repeat Sequences

Z22 and Z19 F A AA
QQ LP NQL ANSPAYLQQ
g repeats L F LV 58-225

C-Terminal Turn

Z22	QQQLLPYNRFSLMNPVLSRQQPIVGGAIF	238-266
Z29QQPPIIGGALF	226-235

Fig. 2-1

3/9

0208418

Fig.2-2

0208418

4/9

Fig. 2-3

3
Fig.

Fig. 4

6/9
248

0208418

NH ₂ -									
c219A2	START :	P	T	T	V	q			
c219B1		L							
c219A81	MAAKIPCLIM	LIGGAGAAT	AQTIPQCSQA	PIASLJPPYL	SPASSCEN	PLIPRPPRQQ	ATIAGILIS	PLFLQQSAL	LQQLPLWILL
c219D1	MAAKIPALLA	LLAISANAVAT	ATIIFQC3Q	*****QYL	SPVTAARPEY	ITPQSYRLQE	ATIASLISL	ALTVQQYAL	LQIQSLVNLN
c219C1	MATKIFSLIM	LLAISACVAN	ATIIFQC5QA	PIASLJPPYL	PSMIAUCEN	PLAQPRUHQ	ATIASNPLS	PLPQQSPAL	***SLVSQL
c219C2									
c219A2		O	H			q			
c219B1				GS	S	*	q		
c219A81	QLVL*****	**ANLAAYSQ	QQQFLPPFQL	AAAMSAAYIQ	QQQLPPFSQL	***AAAYRHI	**QPLPPFQLA	ALASHAYQQ	QQLAPPQLA
c219D1	QQQLPPFQL	VANHLDAYLQ	QQQLPPFQL	AGYNPAAYLQ	AQQQLPPFQL	VISGPAPFLQ	QQLPPFQLQ	VANIAAPLQ	VSPPAAPI,TQ
c219C1	QVLPPLINQ	ALAMISPPGQ	QQQLPPFQL	STLMPAALIQ	QQQLPPFQL	VCHTAPLQ	QQLPPFQLQ	ATLPAAYLQ	VCHTAPLQ
c219C2									
c219A2									
c219B1	P	A	A	V	N	N	T		
c219A81	QQQLPPFQL	APNVOTULLQI	QQQLPPFQL	LTNPAAAYQQ	PLIGGALP				
c219D1	QQQLPPFQLQ	VANNVAFIAQQ	QQQLPPFQLQ	LTMPPTLLQQ	PTIGGAIP				
c219C1	QQQLPPFQLQ	AAAPATLQLQ	QQQLPPFQL	LTDPAAAYQQ	IIIIGCALP				
c219C2									
							-COOH		

Fig. 5

NH₂-

c222A1aa MATKILALLA LLALLVSAVN AFLIPQCSLA PSASISIPQFLP PVTSMGFEIP AVQARYNQLA LAASALQQPI AQLQQQLSLAI LTLQQTIArqq QQQQFLPSLS
c222B1aa S PA S SI A I V
c222C2aa Start: P I L Q V N
c222A1aa HLAMVNPVY LQQQLLAAQ LALANVAAyQ QQQQLQQFMP VLSQLAHVNP AVYL***QIL SSSPLAVGNA PTYLQQQLAQ qIVPALTQLA VAMPAYQQ
c222B1aa A V N L A A QQQ
c222C2aa Q DV A L A A QQQ -COOH
c222A1aa LLPPMQLAVS NSAAAYLQQRQ QLLNPFLAVAN PLVATFLQQQ QQLLPYNQFS LANPAl***QQ PIVGGAIIP
c222B1aa TM A A R V SR
c222C2aa T E P A S SW

7/9

0208418

8/9

Fig. 6

0208418

A

Natural zein mRNA
sequence
c19c1

B

Mutated: @ AA pos. 44
Ile Lys (#41)

C

Mutated: @ AA pos. 167
Asn Lys (#76)

D



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⑯ Publication number:

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A3

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C 12 N 1/20, C 12 N 5/00

㉒ Date of filing: 09.06.86

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㉔ Applicant: LUBRIZOL GENETICS INC., 3375 Mitchell Lane, Boulder Colorado 80301-2244 (US)

㉕ Date of publication of application: 14.01.87
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㉖ Inventor: Larkins, Brian, 2553 Shagbark Lane, West Lafayette Indiana 47906 (US)
Inventor: Cuellar, Richard, 615 Dodge Street, West Lafayette Indiana 47907 (US)

㉗ Designated Contracting States: AT BE CH DE FR GB IT LI LU NL SE

㉘ Representative: Fisher, Adrian John et al, CARPMAELS & RANSFORD 43 Bloomsbury Square, London WC1A 2RA (GB)

㉙ Modified zein.

㉚ Modified seed storage proteins of maize (zeins) containing lysine and genetically modified structural genes encoding these proteins. The structural genes encoding the 19kd and 22kd zein proteins have been genetically modified so that the zeins are modified to contain lysine. These modifications are made without affecting the ability of the modified zeins to function correctly as a storage protein in maize endosperm. Various genetic constructs and vectors comprising these modified zein genes are provided.

EP O 208 418 A3



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Application Number

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EUROPEAN SEARCH REPORT

Page 4
0208418
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EP 86 30 4370

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EUROPEAN SEARCH REPORT

0208418
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